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Article

Are Sugar Concentrations More Important Than Bacterial Identity in Fe(III) Reduction?

Chao Peng, Luwen Zhang, Kuan Cheng, Jin Li, Qiang Zeng, Yundang Wu, Tongxu Liu, Lu Lu,* and Andreas Kappler

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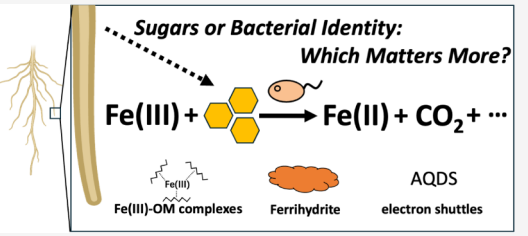
ABSTRACT: Microbial iron(III) (Fe(III)) reduction plays an important role in the environment and is fundamentally driven by the oxidation of organic matter. However, most studies have primarily focused on how different Fe(III) is reduced by different bacteria, while largely overlooking the oxidation side of the reaction. Sugars as primary organic carbon inputs in soils can be utilized by some Fe(III)-reducing bacteria as electron donors. However, the effect of sugar input on microbial Fe(III) reduction remains poorly understood. In this study, we determined Fe(III) reduction kinetics and the extent of three glucose-metabolizing bacteria (*Aeromonas* sp. CD, *Enterobacter* sp. DN, and *Bacillus* sp. GX) at different glucose concentrations. Our results showed a positive correlation between glucose concentrations and the reduction of Fe(III) minerals (ferrihydrite), with significant increases in both the reduction rate and extent observed at low to moderate glucose levels (5−32 mM). However, compared to ferrihydrite, increasing glucose concentrations had a smaller effect on enhancing the reduction rate and extent of Fe(III)-citrate by the three strains. Glucose concentrations also influenced the promoting effect of an electron shuttle (AQDS), which enhanced ferrihydrite reduction at low glucose concentrations (5 mM) but exhibited weaker or even inhibitory effects at higher glucose concentrations (32−65 mM). *Aeromonas* sp. CD, with Mtr-based extracellular electron transfer systems (EET), exhibited higher Fe(III)-citrate reduction rates than the other strains, but the difference in ferrihydrite reduction rates was not as pronounced as in reducing Fe(III)-citrate and ferrihydrite with AQDS. Overall, this study highlights the crucial role of sugars and sugar-metabolizing Fe(III)-reducing bacteria in the iron biogeochemical cycle.



KEYWORDS: *microbial iron reduction, fermentation, ferrihydrite, glucose, electron shuttle*

1. INTRODUCTION

Iron (Fe) plays a crucial role in natural environments.[1] It is an essential element for plant growth, and its bioavailability in soil directly impacts crop health and yield.[2] Additionally, due to the significant differences in solubility and chemical reactivity between Fe(II) and Fe(III), microbial reduction of Fe(III) can regulate the migration and transformation of various elements, as well as organic and inorganic pollutants in soil, through catalysis, precipitation, coprecipitation, and adsorption.[3]

The fate of organic matter is closely related to Fe(III) reduction. On the one hand, Fe(III) reduction can influence the migration, transformation, and bioavailability of organic matter.[4] On the other hand, numerous studies have shown that organic matter with specific functional groups can act as electron shuttles, adsorbents, or chelating agents which have a profound influence on the reduction rate of Fe(III).[3] However, compared to these indirect regulatory functions of organic matter, the primary role of organic matter in microbial Fe(III) reduction is to function as an electron donor, providing reducing power for Fe(III) reduction (Figure 1). Different organic matter can serve as electron donor for microbial Fe(III) reduction, including organic acids,[5] sugars,[6] alcohols,[7] petroleum and aromatic compounds.[8−10]

However, compared to the broad utilization of organic matter by Fe(III)-reducing microorganisms, most previous studies analyzing Fe(III)-reducing bacteria only used non-fermentable organic acids as electron donor. These non-fermentable organic compounds, such as acetate and lactate, can be utilized by some Fe(III)-reducing bacteria, typically the well-studied strains such as *Shewanella oneidensis* MR-1 and *Geobacter sulfurreducens* PCA with extracellular electron transfer systems (EET).[11,12] However, wild-types of these well-studied strains cannot utilize glucose, a typical organic compound used for fermentation.[13,14] In contrast, glucose can be utilized by many less-studied fermentative bacteria that typically lack the EET for Fe(III) reduction.[15−18] Although previous studies have shown that those fermentative bacteria do not gain significant energetic advantages from Fe(III) reduction and do not rely on it for growth,[11] Fe(III) reduction

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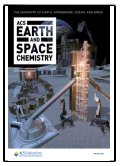

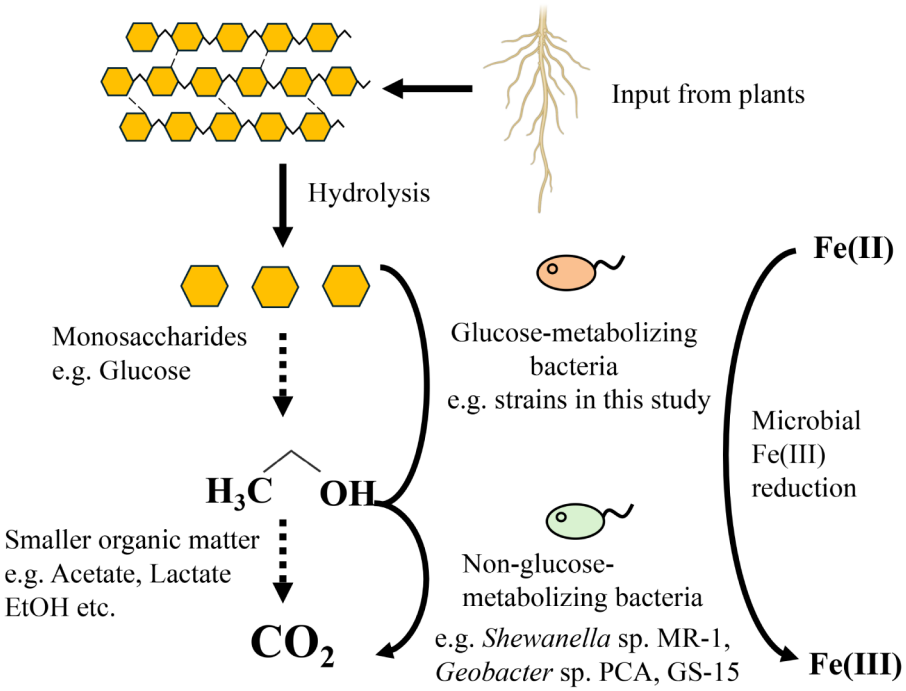

Figure 1. Illustration of microbial Fe(III) reduction processes driven by plant-derived sugars. Glucose is a typical monosaccharide derived from the hydrolysis of plant-derived polysaccharides in the soil. It can be utilized by glucose-metabolizing bacteria, some of which have the ability to reduce Fe(III) during fermentation.

was thought to help these fermentative bacteria to maintain environmental conditions (e.g., pH) and enhance the decomposition of fermentation substrates.[18−20]

Although studies on the effects of sugar as an electron donor on Fe(III)-reducing bacteria are limited, previous studies suggested that their contribution to Fe(III) reduction may still be significant. Because, first and most importantly, plant-derived sugars are the primary source of organic carbon input in soil.[21] Due to the dominance of polysaccharides in plants (50−70% of dry mass),[21] a large amount of the sugars (about 20−30% of the total carbon assimilated through plant photosynthesis) were estimated to be transferred into soil every year.[22] In addition to agricultural habitats, many other environments, such as wastewater, were also found to contain highly abundant fermentable organic compounds,[23] which can be utilized by fermentative bacteria to reduce Fe(III) to Fe(II). Second, the taxa of sugar-metabolizing fermentative bacteria with Fe(III)-reducing capacity are highly diverse and include common bacteria such as *Escherichia coli* and *Clostridium pasteurianum*.[16,24] In contrast, bacteria with Fe(III)-reducing EET constitute only a small fraction of the total bacterial community in the environments.[25,26] Third, although the well-studied Fe(III)-reducing bacteria, *Shewanella oneidensis* MR-1 and *Geobacter sulfurreducens* PCA, can utilize nonfermentable small organic acids for Fe(III) reduction, parts of the small organic acids they use are in fact products of the fermentation process catalyzed by sugar-metabolizing bacteria[27,28] (Figure 1). Last but not least, once fermentative Fe(III)-reducing bacteria utilized plant-derived sugar to reduce Fe(III), there may be little to no labile Fe(III) left for nonglucose-metabolizing Fe(III)-reducing bacteria.

However, how the input of sugars influences the rates and extent of microbial Fe(III)-reduction still remains largely unclear. In this study we selected three glucose-metabolizing bacteria: including a Gram-positive strain lacking an outer membrane (*Bacillus* sp. GX), a Gram-negative strain with a complete Mtr-based EET (*Aeromonas* sp. CD),[29] and a Gram-negative strain without previous identified EET genes (*Enterobacter* sp. DN). Through microbial Fe(III) reduction experiments and comparative genomics under identical conditions, we examined the effect of glucose concentrations on the microbial reduction of dissolved Fe(III)-OM complexes (Fe(III)-citrate) and solid Fe(III) minerals (ferrihydrite) with and without an electron shuttle (AQDS). The study aims to address the following scientific questions: under the input of glucose, a typical monosaccharide widely utilized by bacteria and derived from the hydrolysis of polysaccharides input by plants into the soil, how do bacterial Fe(III) reduction and the role of electron shuttles respond, and do different bacteria with and without Fe(III)-reducing EET behave differently in these processes.

2. MATERIALS AND METHODS

2.1. Source of Bacteria. The glucose-metabolizing Fe(III)-reducing bacterium with Mtr-based EET, *Aeromonas* sp. CD was previously isolated from agricultural soil.[29] The Gram-negative and Gram-positive fermentative bacteria without previous identified EET for Fe(III) reduction but with Fe(III)-reducing capacity, *Enterobacter* sp. DN and *Bacillus* sp. GX were isolated from lake sediments and rhizosphere soil, respectively within the China West Normal University campus (GPS: 30°49′7″N, 106°3′50″E). For strains GX and CD, 10 mM sodium lactate and 10 mM ferrihydrite were added to the enrichment medium,[30] whereas 10 mM sodium acetate and 10 mM Fe(III)-citrate were used for strain DN. Ferrihydrite was synthesized using $Fe(NO_3)_3{\cdot}9H_2O$ and KOH following a previously described protocol.[31] After synthesis, the ferrihydrite was suspended in anoxic H_2O in a glovebox (100% N_2). Fe(III)-citrate stock solution (500 mM) was prepared by dissolving the $FeC_6H_5O_7$ salts and adjusting the pH to 7.0 with

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NaOH. After synthesis, Fe(III)-citrate solutions were boiled and cooled under N_2 to remove oxygen. The cultures were incubated at 28 °C until Fe(III) reduction was observed by eye (change in color from brown to black), after which they were transferred to fresh medium. The enriched cultures were spread on LB agar plates containing 1.5% agar (strain GX and DN) or anoxic freshwater medium plates containing 1.5% agar and 10 mM Fe(III)-citrate (strain CD). The isolated strains were preserved in 50% glycerol at −80 °C. The purity of strain DN and GX was confirmed via 16S rRNA sequencing, identifying the strains as belonging to the genera *Enterobacter* and *Bacillus*, respectively. The phylogenetic analysis of the 16S rRNA gene of the studied bacteria is presented in Figure 2, which includes the three strains investigated (*Aeromonas* sp. CD, *Enterobacter* sp. DN, *Bacillus* sp. GX) and reference 16S rRNA gene sequences from strains such as *Shewanella oneidensis* MR-1 and *Geobacter sulfurreducens* PCA.

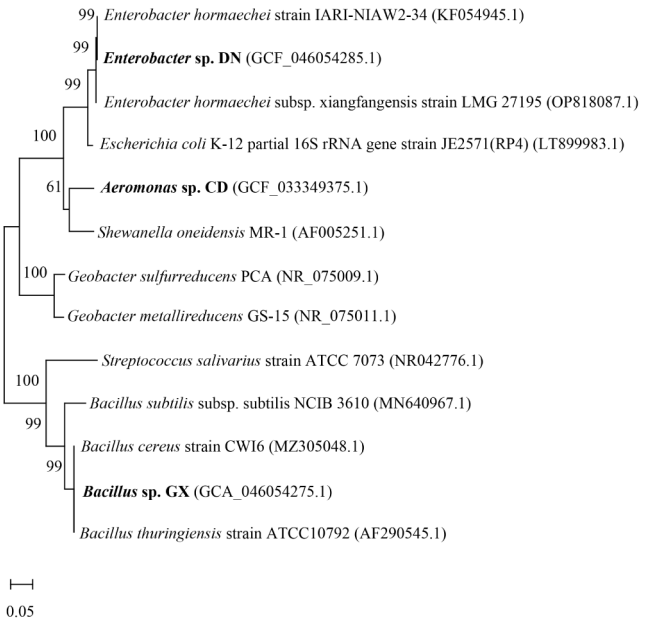

Figure 2. Phylogenetic analyses of 16S rRNA gene sequences from bacterial strains examined in this study (strains used in this study are shown in bold) and related reference strains. Phylogenetic trees were constructed using MEGA7 with the maximum likelihood method and 1,000 bootstrap replications. Bootstrap values are indicated along branch points.

2.2. Cell Suspension Preparation and Enumeration. The three bacteria were inoculated separately into LB liquid medium and cultured aerobically at 28 °C until they reached the exponential growth phase (Figure S1). Cells were harvested by centrifugation (3020 g, 5 min) and washed three times with anoxic 20 mM PIPES buffer (pH 7). Then the cells were resuspended in the same buffer. To quantify viable cells, the prepared cell suspensions were diluted and evenly spread onto LB agar plates in triplicate. After oxic incubation at 25 °C for 24 h, colony-forming units (CFU) were manually counted.

2.3. Iron(III) Reduction Experiments under Different Carbon Source Conditions. To investigate the effect of sugar input on Fe(III) reduction rates, modified basal media were used containing 100 mM PIPES (pH 7), 0.6 g·L^{-1} KH_2PO_4, 0.3 g·L^{-1} NH_4Cl, 0.025 g·L^{-1} $MgSO_4·7H_2O$, 0.187 g·L^{-1} $MgCl_2$, 0.1 g·L^{-1} $CaCl_2·2H_2O$. Initial inoculum concentrations were set at 5.5×10^8, 2.8×10^9 and 1.8×10^9 CFU/mL for strains CD, DN and GX. Experimental variables included glucose concentrations (5, 32, and 65 mM), which fall within the typical range found in the environment (in grams per kilogram of soil),[21] Fe(III) source types (10 mM Fe(III)-citrate or ferrihydrite), and the presence or absence of an electron shuttle (0.1 mM AQDS) for ferrihydrite reduction.

Cell suspensions were dispensed into sealed butyl rubber-stoppered serum bottles with varying experimental setups and incubated at 28 °C in the dark. No Fe(III) reduction was observed in abiotic controls (Figure S2). Each experimental condition was set with triplicates. Butyl stoppers and glassware and were sterilized by autoclaving at 121 °C or dry heating at 180 °C.

2.4. Iron(III) Reduction Kinetics. Samples were collected using sterile syringes preflushed with N_2. Collected samples were mixed with 1 M HCl immediately, and incubated in the dark for 2 h to extract Fe(II). Sampling frequency ranged from several hours to several days, depending on the Fe(III) reduction rate under different glucose concentrations and bacterial strains. Quantification of Fe(II) was performed by mixing 1 M HCl with 0.1% ferrozine solution (dissolved in 50% w/v ammonium acetate). Standard curves were prepared using $(NH_4)_2Fe(SO_4)_2·6H_2O$ dissolved in 1 M HCl. Reduction rates of Fe(III) were calculated using linear regression based on data points taken before Fe(III) reduction reached the equilibrium phase.

2.5. Genomic Sequencing and Functional Annotation. Draft genome sequencing of strains GX and DN was performed on the Illumina NovaSeq 6000 platform (Majorbio, Shanghai), and data were processed using Fastp[32,33] and SOAPdenovo2[34] for quality control and assembly. The complete genome of strain CD was sequenced using both Illumina NovaSeq 6000 and Nanopore PromethION platforms. Hybrid assembly of short and long reads was conducted using Unicycle v0.4.8[35] to generate complete genomes. Fe(III) reduction-related genes were identified using FeGenie.[36] Assembled genome data have been submitted to NCBI under accession numbers PRJNA1029467 for strain CD, and PRJNA1188212 for both strain DN and GX.

3. RESULTS

3.1. Effects of Glucose Concentration on Reduction of Ferrihydrite without AQDS. The effects of glucose concentration on rates and extents of Fe(III) reduction were determined by performing microbial Fe(III) reduction experiments. Generally, the results of the experiments showed a positive correlation between glucose concentration and the rates and extent of ferrihydrite reduction for all three strains (Figures 3, 4, Table 1). In addition, at lower glucose concentrations (5−32 mM), the influence of glucose concentration on Fe(III) reduction was more significant, whereas this effect diminished at medium to high glucose concentrations (32−65 mM).

Specifically, when glucose concentrations increased from 5 to 32 mM, the reduction rates of ferrihydrite by strains CD, DN, and GX increased by 21, 7, and 3.6 times, respectively, from 0.02, 0.02, and 0.07 mM/day to 0.42, 0.14, and 0.25 mM/day (Figure 4). However, further increasing the concentration to 65 mM resulted in smaller increases in reduction rates by 1.4, 1.4, and 1.3 times, to 0.59, 0.20, and 0.32 mM/day, respectively.

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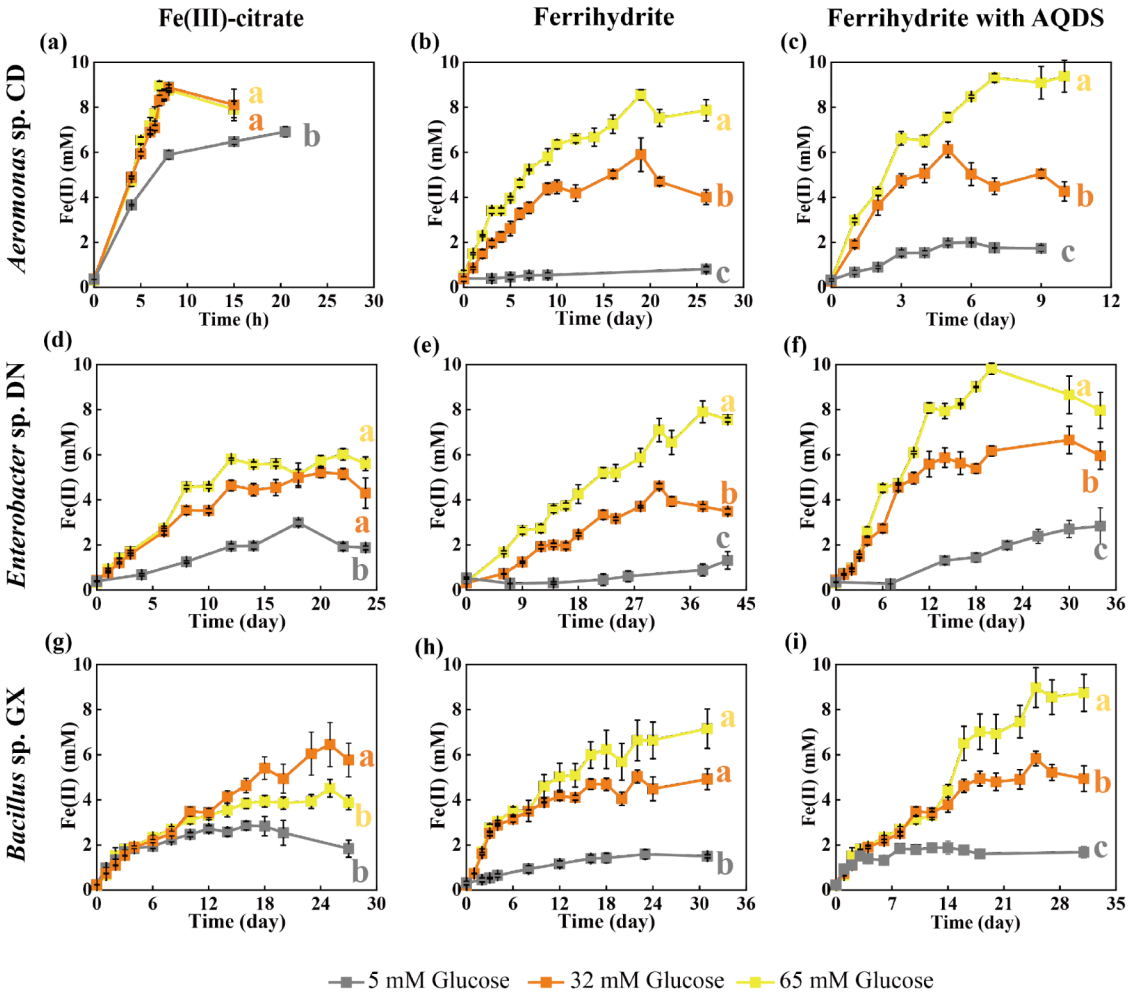

Figure 3. Fe(II) production over time during the reduction of Fe(III)-citrate and Ferrihydrite (with and without AQDS) by *Aeromonas* sp. CD, *Enterobacter* sp. DN, and *Bacillus* sp. GX under three glucose concentrations (5, 32, and 65 mM). Different colored letters within each panel indicate results from one-way ANOVA with posthoc Tukey HSD test for Fe(III) reduction rates, corresponding to the sample color (gray for 5 mM, orange for 32 mM, yellow for 65 mM). Samples sharing the same letter are not significantly different ($p > 0.05$), while different letters indicate significant differences ($p < 0.05$). Error bars represent the standard error of three biological replicates.

The extent of Fe(III) reduction also increased with rising glucose concentrations, although the increase was less pronounced compared to the enhancement in Fe(III) reduction rates. When the glucose concentration was increased from 5 to 32 mM, the extent of ferrihydrite reduction by strains CD, DN, and GX increased by 7.4, 3.5, and 3.0 times, reaching 5.9 ± 0.7, 4.6 ± 0.1, and 4.9 ± 0.5 mM, respectively. However, when the glucose concentration was further increased from 32 to 65 mM, the extent of ferrihydrite reduction by strains CD, DN, and GX only increased by 1.5, 1.7, and 1.5 times, respectively (Table 1), with nearly all Fe(III) being reduced.

3.2. Influence of Glucose Concentration on the Effect of AQDS on Ferrihydrite Reduction. The effect of an electron shuttle (AQDS) on ferrihydrite reduction was evaluated at three different glucose concentrations. Our results showed that AQDS promoted ferrihydrite reduction across all glucose concentrations, however, its promoting effect diminished as glucose concentration increased.

At the lowest tested glucose concentration (5 mM), AQDS exhibited the most significant promoting effect on microbial Fe(III) reduction. Compared to controls without AQDS, the reduction rates of ferrihydrite with AQDS by strains CD, DN, and GX increased by 16.5, 4.5, and 2.1 times, reaching 0.33, 0.09, and 0.15 mM/day (Figure 5), respectively. The reduction extent of ferrihydrite with AQDS by strains CD, DN, and GX also increased 2.5, 2.2, and 1.2 times, reaching 2.0 ± 0.02, 2.8 ± 0.8 and 1.9 ± 0.2 mM (Table 1) respectively. However, for strains CD, DN and GX, when the glucose concentration was 32 mM, their reduction rates of ferrihydrite with AQDS were 1.27, 0.41, and 0.22 mM/day. These corresponded to 3.0-, 2.9-, and 0.9-fold of the rates observed without AQDS. Under this condition, AQDS did not increase the rate of ferrihydrite reduction by strain GX. When the glucose concentration was increased to 65 mM, AQDS still enhanced the extent of ferrihydrite reduction by 3.4 and 2.6 times for strains CD and DN, respectively, but did not promote ferrihydrite reduction by strain GX.

The extents of ferrihydrite reduction with AQDS followed a similar trend. At a glucose concentration of 5 mM, the extent of ferrihydrite reduction by strains CD, DN, and GX with AQDS was 2.5, 2.2, and 1.2 times higher, respectively, than the setups without AQDS (Table 1). At 32 mM glucose, the extent of ferrihydrite reduction with AQDS reached 6.1 ± 0.3, 6.7 ± 0.6, and 5.8 ± 0.3 mM for strains CD, DN, and GX, respectively, which were only 1.03, 1.46, and 1.18 times higher

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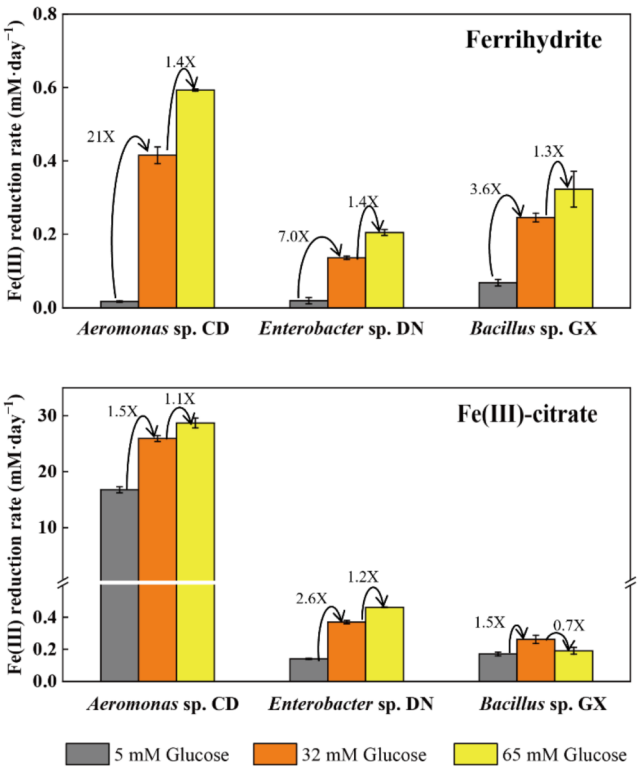

Figure 4. Ferrihydrite and Fe(III)-citrate reduction rates by *Aeromonas* sp. CD, *Enterobacter* sp. DN, and *Bacillus* sp. GX under three glucose concentrations (5, 32, and 65 mM). Error bars represent the standard error of three biological replicates. Arrows and the numbers above the bars indicate the relative differences in reduction rates between the glucose concentrations, highlighting the effect of glucose concentration on microbial Fe(III) reduction. The result showed that microbial Fe(III) reduction is greatly influenced by glucose concentration, and Fe(III)-reducing bacteria possessing EET exhibit much higher reduction rates of Fe(III)-citrate compared to those lacking EET.

than the setups without AQDS (Table 1). At 65 mM glucose, with nearly all the ferrihydrite reduced in the setups without AQDS, AQDS had almost no effect on the extent of ferrihydrite reduction for any of the strains tested.

3.3. Effects of Glucose Concentration on Reduction of Dissolved Fe(III). The ability of the strains to reduce Fe(III)-citrate was also determined at three different glucose concentrations. Unlike the reduction of ferrihydrite, the reduction of Fe(III)-citrate was less affected by glucose concentration. When the glucose concentration was increased from 5 to 32 mM, the reduction rates of Fe(III)-citrate by strains CD, DN, and GX increased by only 1.5, 2.6, and 1.5 times, respectively, from 16.8, 0.14, and 0.17 mM/day to 25.9, 0.37, and 0.26 mM/day. When the glucose concentration further increased from 32 to 65 mM, only slight changes were observed in the Fe(III)-citrate reduction rates of strains CD and DN, with increases of 1.1 and 1.2 times, respectively. The reduction rate of Fe(III)-citrate by strain GX even decreased to 0.19 mM/day (Figures 3, 4).

The extent of Fe(III)-citrate reduction also exhibited a pattern consistent with changes in Fe(III) reduction rates. When the glucose concentration increased from 5 to 32 mM, the extent of Fe(III)-citrate reduction by strains CD, DN, and GX increased by only 1.3, 1.8, and 2.2 times, reaching 8.8 ± 0.1, 5.2 ± 0.2, and 6.4 ± 0.8 mM, respectively (Table 1), with nearly all the Fe(III)-citrate reduced by strain CD. When the glucose concentration was further increased from 32 to 65 mM, the extent of Fe(III)-citrate reduction by strains CD and DN did almost not increase, while the extent of Fe(III)-citrate reduction by strain GX even decreased to 4.5 ± 0.4 mM.

In addition to the differences observed at varying glucose concentrations, significant differences were also observed in the reduction rates of Fe (III)-citrate among the three Fe(III)-reducing bacteria. For strain CD, the overall reduction rates of Fe(III)-citrate ranged from 16.8 to 28.7 mM/day at all carbon source concentrations, which were up to 120 and 151 times faster than those of strain DN and strain GX under the same conditions (Table 1).

3.4. Genes in Fe(III) Reduction. Genomic sequencing revealed that only strain CD possesses the *mtrCAB* gene cluster which encodes the extracellular electron transfer systems between inner and outer membrane.[37] Although strain GX and strain DN showed Fe(III) reduction capabilities with glucose, neither strain contained any known cytochrome c-related genes for Fe(III)-reduction, confirming their classification as fermentative iron-reducing bacteria. The results are summarized in Table S1.

4. DISCUSSION

4.1. Sugar Availability as a Key Regulator of Microbial Fe(III) Reduction. Interestingly, the results of this study demonstrated that, regardless of whether bacteria possess an Mtr-based EET or not, the Fe(III)-reducing capacity of all the three bacteria tested was influenced by the concentration of the sugar present. These microbial-level findings align with patterns observed in paddy soils where a correlation between the concentration of glucose, abundance of glucose-fermenting bacteria (*Clostridium* sp.) and Fe(III) reduction was reported.[38] Similarly, a correlations between the abundance of Fe(III)-reducing bacteria and TOC in marine and lake sediments have also been reported.[39,40] As many bacteria in the environment lack specific EET for Fe(III) reduction,[26] these findings would raise an intriguing question: could the concentrations of sugar as an electron donor be more critical than the identity of bacterial species involved in microbial Fe(III) reduction in the environment?

From the perspective of abundance and diversity of bacteria with and without EET for Fe(III) reduction, this appears to be true. Unlike other microbial biogeochemical processes, such as nitrate or sulfate reduction, which rely on specific terminal reductases,[41] the fermentative bacteria lacking an EET for Fe(III) reduction, such as strains DN and GX used in this study, were still capable of reducing ferrihydrite and Fe(III)-citrate when supplied with glucose (Figure 3, Table 1). Notably, even *Escherichia coli* has been recognized as an Fe(III)-reducer,[16,24] and the ability of fermentative bacteria to reduce Fe(III) was identified long before the discovery of Fe(III)-reducing bacteria with EET.[42] Given that Fe(III)-reducing bacteria with EET may not be highly abundant in many environments, even in those with high Fe(III) availability,[26,43] the findings of this study suggest that when plant-derived carbon is introduced into the environment, a much broader range of bacterial genera, beyond a few specific species with extracellular electron transfer chains, may all contribute to Fe(III) reduction.

In addition, plant-derived sugars, such as glucose, not only regulated ferrihydrite reduction but also appeared to influence the effect of electron shuttles, which have been identified as

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Table 1. Summary of Genes Related to Fe(III) Reduction in Each Strain, the Maximum Extent of Fe(III) Reduction, the Minimum Percentages of Electron Donors Used for Fe(III) Reduction, Overall Fe(III) Reduction Rates under Different Experimental Conditions in This Study[a]

Bacteria and Fe(III) reduction genes	E^- acceptor	Glucose (mM)	Fe(III) reduced (mM)	Min. e^- to Fe(III) (%)	Fe(III) red. rate (mM/d)
Aeromonas sp. CD (with *mtrCAB* gene cluster)	Fe(III)-citrate	5	6.9 ± 0.3	5.75	16.8
		32	8.8 ± 0.1	1.14	25.9
		65	8.9 ± 0.2	0.57	28.7
	Ferrihydrite	5	0.8 ± 0.1	0.67	0.02
		32	5.9 ± 0.7	0.76	0.42
		65	8.6 ± 0.2	0.56	0.59
	Ferrihydrite and AQDS	5	2.0 ± 0.02	1.67	0.33
		32	6.1 ± 0.3	0.79	1.27
		65	9.3 ± 0.2	0.6	2.02
Enterobacter sp. DN (no specific Fe(III) reduction genes identified)	Fe(III)-citrate	5	2.9 ± 0.1	2.42	0.14
		32	5.2 ± 0.2	0.67	0.37
		65	6.0 ± 0.3	0.39	0.46
	Ferrihydrite	5	1.3 ± 0.4	1.08	0.02
		32	4.6 ± 0.1	0.59	0.14
		65	7.9 ± 0.5	0.51	0.2
	Ferrihydrite and AQDS	5	2.8 ± 0.8	2.33	0.09
		32	6.7 ± 0.6	0.87	0.41
		65	9.8 ± 0.2	0.63	0.52
Bacillus sp. GX (no specific Fe(III) reduction genes identified)	Fe(III)-citrate	5	2.9 ± 0.1	2.42	0.17
		32	6.4 ± 0.8	0.83	0.26
		65	4.5 ± 0.4	0.29	0.19
	Ferrihydrite	5	1.6 ± 0.2	1.33	0.07
		32	4.9 ± 0.5	0.63	0.25
		65	7.2 ± 0.8	0.47	0.32
	Ferrihydrite and AQDS	5	1.9 ± 0.2	1.58	0.15
		32	5.8 ± 0.3	0.75	0.22
		65	8.9 ± 0.8	0.57	0.32

[a]The minimum percentages of electron donors used for Fe(III) reduction were calculated based on the assumption that glucose oxidation completely produces CO_2.

key factors in Fe(III)-reducing bacteria both with and without EET.[44,45] Results of our study demonstrated that AQDS cannot be equally effective at high glucose concentrations (32 and 65 mM) as it is at low concentrations (5 mM). At high glucose concentrations, the addition of AQDS did not enhance Fe(III) reduction by *Bacillus* sp. GX, and the rate remained similar to that without AQDS. This point may be particularly important to consider when using material with electron shuttling capabilities, e.g., biochar,[46,47] to regulate microbial Fe(III) reduction processes in the environment with sugar input, e.g., agricultural soil.

4.2. Sugar Over Species? Maybe Not Always. However, on the other hand, bacterial species may also be more critical than sugar in certain environments. For instance, in environments with limited or no continuous input of sugar, such as deep subsurface sediments or permafrost regions,[48,49] non-fermentative Fe(III)-reducing bacteria, e.g., *Geobacter* sp. which have capacity to utilize nonfermentable organic acids, may play a more significant role than the bacteria which depends on the fermentation of sugars.

Furthermore, in environments with abundant electron shuttles and Fe(III)-OM complexes, Fe(III)-reducing bacteria with EET may still outcompete the sugar-metabolizing fermentative bacteria without EET. In our study, bacteria with Mtr-based EET (strain CD) exhibited higher Fe(III) reduction rates than the other two bacteria without EET (strain GX and DN) across all conditions, despite having a lower initial inoculum. This difference became even more pronounced during the reduction of Fe(III)-citrate or ferrihydrite in the presence of AQDS. Specifically, ferrihydrite reduction rates by strain CD were only up to 3-fold higher than those of strain DN, and ranged from similar to up to 1.8-fold higher than those of strain GX. However, strain CD reduced Fe(III)-citrate at rates 120-fold and 151-fold higher than strains DN and GX, respectively (Table 1). Although direct comparisons of absolute performance among strains may be inappropriate due to potential differences in their physiological adaptability to laboratory conditions, these findings emphasize that the magnitude of interstrain differences is strongly influenced by organic matter. This includes both glucose, which serves as an electron donor, and organic ligands, which can alter the speciation of Fe(III).

Additionally, although all tested strains showed a consistent trend in Fe(III) reduction rates and extent in response to glucose input, the degree of change varied among them. Compared to the two Gram-negative strains, the Gram-positive Fe(III)-reducing bacterium *Bacillus* sp. GX exhibited a much weaker enhancement or even inhibition of Fe(III)-citrate reduction with increasing glucose concentrations (Figure 4), and the addition of AQDS did not promote ferrihydrite reduction at higher glucose concentrations (Figure 5). This could be probably due to the difference in cell wall structure, sugar transport mechanisms and other metabolic factors.[41] For example, the carbon catabolite repression (CCR) system and

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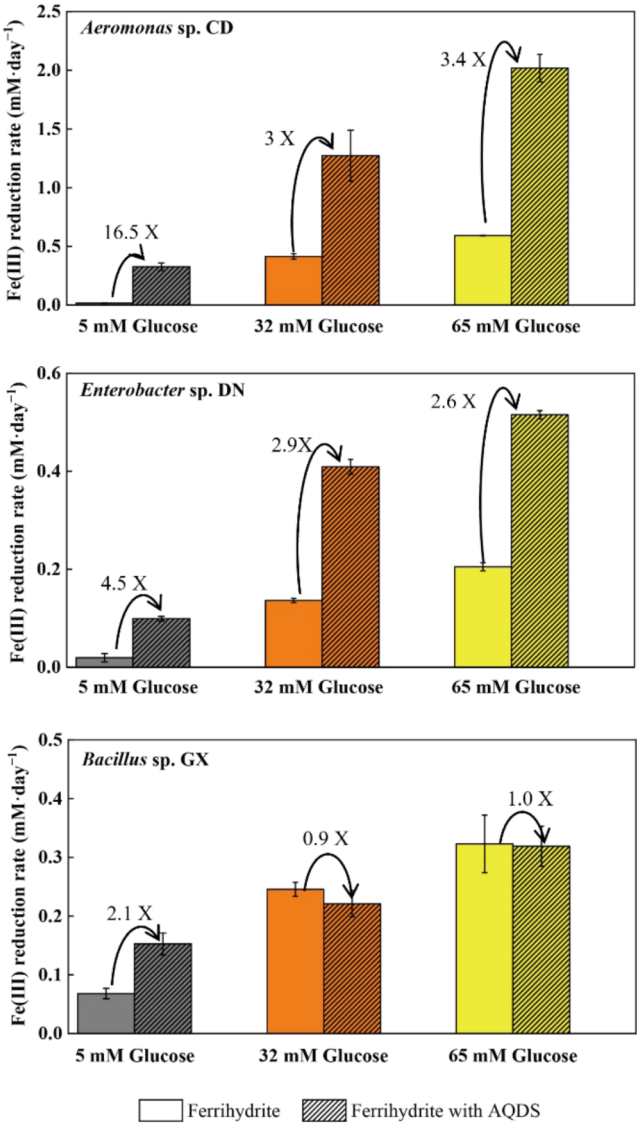

Figure 5. Ferrihydrite reduction rates with and without AQDS by *Aeromonas* sp. CD, *Enterobacter* sp. DN, and *Bacillus* sp. GX under three glucose concentrations (5, 32, and 65 mM). Error bars represent the standard error of three biological replicates. Arrows and the numbers above the bars indicate the relative differences in Fe(III) reduction rates under the same glucose concentration, highlighting the impact of AQDS on microbial Fe(III) reduction at different glucose concentrations. The results show that the promoting effect of AQDS on microbial Fe(III) reduction rates is more pronounced under low-glucose conditions.

phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS) in Gram-positive bacteria like *Bacillus* differ significantly from those in Gram-negative bacteria, potentially leading to different glucose utilization efficiency and upper limit under identical conditions.[50,51] This limitation in glucose utilization may further contribute to the observed upper limit of Fe(III) reduction rates for both Fe(III)-citrate and ferrihydrite with AQDS by *Bacillus* sp. GX, which remained about 0.2 to 0.3 $mM{\cdot}day^{-1}$ across different glucose concentrations (Figures 4 and 5). Thus, when evaluating the relative contribution of Gram-positive and or Gram-negative bacteria on Fe(III) reduction in different environments,[43] factors such as the concentration of glucose, Fe(III)-chelators and electron shuttles should all be taken into consideration.

Besides, our results also revealed that only a small fraction of the electrons was transferred to Fe(III), and the proportion of electrons directed toward Fe(III) decreased as glucose concentration increased (Table 1). This effect was even more pronounced when Fe(III)-citrate was used as the electron acceptor or when AQDS was present at high glucose concentrations. For example, as glucose concentration increased from 5 to 32 mM, the minimum percentage of electrons transferred to Fe(III)-citrate by strain CD decreased from 5.8 to 1.1% (Table 1). This suggested that in natural environments, as sugar concentrations increase, those bacteria may prefer to direct even more electrons toward fermentation rather than to Fe(III) minerals, similar to what has been observed with *E. coli* under aerobic conditions.[52] And this result also implies that optimizing the electron transfer of glucose-fermentation-derived electrons to Fe(III) minerals holds great potential for regulating Fe(III) reduction processes, highlighting the importance of further investigation.

5. CONCLUSIONS

The results of this study suggested that the input of sugar has a significant impact on microbial Fe(III) reduction processes in the environment. The reducing rates and extent of Fe(III) minerals and Fe(III)-OM complexes by the three types of bacteria were enhanced by increasing glucose concentrations. This effect was stronger for ferrihydrite reduction at low to moderate glucose concentrations (5−32 mM) than at higher glucose concentrations (32−65 mM) or for the reduction of Fe(III)-OM complexes. Additionally, input of sugar also decreased the promoting effect of an electron shuttle (AQDS) on ferrihydrite reduction.

In this study, bacteria with or without Mtr-based EET could all reduce Fe(III) using glucose as substrate, and exhibited similar response to glucose input (Figure 3). However, in the environment with electron shuttles and Fe(III)-chelators, bacteria with EET may have even more competence in reducing Fe(III) than fermentative bacteria without EET, as their Fe(III)-OM reduction rates could be more than 2 orders of magnitude higher than bacteria without EET (Figure 4). Although the glucose concentrations in natural environments may not always reach the maximum level used in this study (65 mM), previous studies have reported the glucose-metabolizing hotspots in specific microenvironments such as the rhizospheres.[53] These regions are also critical zones where microbial Fe(III) reduction can significantly influence processes relevant to humans and other organisms through the food chain, such as the immobilization and mobility of heavy metals. Overall, result of this study demonstrated that sugars may play a more crucial role in microbial Fe(III) reduction than previously thought. Future research on microbial iron cycling processes in the environment with potential input of sugars, such as agricultural soils, should focus more on the glucose-metabolizing fermentative bacteria and the concentration of sugars, rather than solely considering bacteria with EET and organic matter as electron shuttles and chelators.

■ ASSOCIATED CONTENT

Supporting Information

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AUTHOR INFORMATION

Corresponding Author

Lu Lu − *Key Laboratory of Southwest China Wildlife Resources Conservation, College of Life Sciences, China West Normal University, Nanchong 637000, China; College of Environmental Science and Engineering, China West Normal University, Nanchong 637000, China*; Email: llu327@cwnu.edu.cn

Authors

Chao Peng − *Key Laboratory of Southwest China Wildlife Resources Conservation, College of Life Sciences, China West Normal University, Nanchong 637000, China; Precise Synthesis and Function Development Key Laboratory of Sichuan Province, College of Chemistry and Chemical Engineering, China West Normal University, Nanchong 637000, China*; orcid.org/0000-0003-3433-4319

Luwen Zhang − *Key Laboratory of Southwest China Wildlife Resources Conservation, College of Life Sciences, China West Normal University, Nanchong 637000, China*

Kuan Cheng − *National-Regional Joint Engineering Research Center for Soil Pollution Control and Remediation in South China, Guangdong Key Laboratory of Integrated Agro-environmental Pollution Control and Management, Institute of Eco-environmental and Soil Sciences, Guangdong Academy of Sciences, Guangzhou 510650, China*

Jin Li − *Key Laboratory of Southwest China Wildlife Resources Conservation, College of Life Sciences, China West Normal University, Nanchong 637000, China*

Qiang Zeng − *Center for Geomicrobiology and Biogeochemistry Research, State Key Laboratory of Biogeology and Environmental Geology and School of Earth Sciences and Resources, China University of Geosciences, Beijing 100083, China*; orcid.org/0000-0002-7766-3588

Yundang Wu − *National-Regional Joint Engineering Research Center for Soil Pollution Control and Remediation in South China, Guangdong Key Laboratory of Integrated Agro-environmental Pollution Control and Management, Institute of Eco-environmental and Soil Sciences, Guangdong Academy of Sciences, Guangzhou 510650, China*; orcid.org/0000-0003-2996-5407

Tongxu Liu − *National-Regional Joint Engineering Research Center for Soil Pollution Control and Remediation in South China, Guangdong Key Laboratory of Integrated Agro-environmental Pollution Control and Management, Institute of Eco-environmental and Soil Sciences, Guangdong Academy of Sciences, Guangzhou 510650, China*; orcid.org/0000-0002-2348-3952

Andreas Kappler − *Geomicrobiology Group, Department of Geosciences, University of Tübingen, Tübingen 72076, Germany*; orcid.org/0000-0002-3558-9500

Complete contact information is available at: https://pubs.acs.org/10.1021/acsearthspacechem.5c00094

Notes

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